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Yue Y; Widmer D A; Halladay A K; Cerretti D P; Wagner G C; Dreyer J L; Zhou R Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin -B2. JOURNAL OF NEUROSCIENCE, (1999 Mar 15) 19 (6) 2090-101.

Gale, Nicholas W.; Yancopoulos, George D. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, Angiopoietins, and ephrins in vascular development Genes Dev. (1999), 13(9), 1055-1066

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Gerety S S; Wang H U; Chen Z F; Anderson D J Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B in cardiovascular development. MOLECULAR CELL, (1999 Sep) 4 (3) 403-14.

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Symmetrical Mutant Phenotypes of the Receptor *EphB4* and Its Specific Transmembrane Ligand *ephrin-B2* in Cardiovascular Developm nt

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Summary

Ephrin-B2 is a transmembrane ligand that is specifically expressed on arteries but not veins and that is essential for cardiovascular development. However, ephrin-B2 is also expressed in nonvascular tissues and interacts with multiple EphB class receptors expressed in both endothelial and nonendothelial cell types. Thus, the identity of the relevant receptor for ephrin-B2 and the site(s) where these molecules interact to control angiogenesis were not clear. Here we show that EphB4, a specific receptor for ephrin-B2, is exclusively expressed by vascular endothelial cells in embryos and is preferentially expressed on veins. A targeted mutation in *EphB4* essentially phenocopies the mutation in *ephrin-B2*. These data indicate that ephrin-B2–EphB4 interactions are intrinsically required in vascular endothelial cells and are consistent with the idea that they mediate bidirectional signaling essential for angiogenesis.

Introduction

The assembly of the embryonic circulatory system presents a fascinating problem in genetics and cell biology that is relevant to both development and disease. The cardiovascular system is the first organ system to form during embryogenesis. Beginning on about day 8 of gestation (E8.0) as the heart starts to beat, individual angioblasts assemble into a primitive capillary plexus, in a process known as vasculogenesis (reviewed in Risau and Flamme, 1995). Over the next 24–36 hr, this plexus undergoes a remarkable morphogenetic transformation, termed angiogenesis, in which it is remodeled into an intricately branched network (reviewed in Risau, 1997). This rapid and dynamic process involves both capillary remodeling and recruitment of smooth muscle cells to form the external walls of the vasculature (Folkman and D'Amore, 1996). It is also coordinated with the establishment of blood flow.

The cellular and molecular mechanisms underlying vasculogenesis and angiogenesis are still poorly understood. However, an increasing number of intercellular signaling molecules have been identified that play an essential role in this process. Prominent among these are transmembrane receptor tyrosine kinases (RTKs) and their ligands (reviewed in Hanahan, 1997; Gale and Yancopoulos, 1999). These include the vascular endothelial growth factors (VEGFs) and their receptors, which are essential for vasculogenesis (Fong et al., 1995; Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996), PDGF-B and its receptors (Benjamin et al., 1998; Hirschi et al., 1998), and the recently discovered angiopoietins (Davis et al., 1996) and their receptors, which are critical for angiogenesis (Sato et al., 1995; Suri et al., 1996). How these different signaling systems functionally interact, and the cellular processes they control, is not yet clear. Nevertheless, manipulation of these signaling systems has already been employed as a new approach to therapeutic intervention in several important clinical settings, such as cancer (Folkman, 1998a) and heart disease (Folkman, 1998b).

Eph receptors, which comprise the largest family of RTKs, and their membrane-associated ligands, the ephrins (reviewed in Gale et al., 1996), have also been implicated in angiogenesis (Pandey et al., 1995; Stein et al., 1998). Recently, ephrin-B2, a transmembrane ligand (Bennett et al., 1995; Bergemann et al., 1995), was shown to be essential for angiogenesis and cardiac development in vivo (Wang et al., 1998). The phenotype of *ephrin-B2* homozygous mutant embryos was superficially similar to that of other RTK ligands required for angiogenesis (Sato et al., 1995; Suri et al., 1996). Remarkably, however, in contrast to these other ligands that are uniformly expressed within the circulatory system, *ephrin-B2* is specifically expressed by arteries but not veins (Wang et al., 1998; Adams et al., 1999) while EphB4, one of its receptors (Brambilla et al., 1995; Sakano et al., 1996), is expressed conversely on veins but not arteries (Wang et al., 1998; Adams et al., 1999). These data provided one of the first examples of a genetic distinction between these two vessel subtypes and suggested that ephrin-mediated interactions between them may be essential for angiogenesis.

These observations left several important issues unresolved, however. First, although artery specific within the circulatory system, ephrin-B2 is expressed by several other nonvascular tissues, many of which contact developing blood vessels (Bergemann et al., 1995; Wang and Anderson, 1997; Wang et al., 1998). This raised the question of whether the essential angiogenic function of ephrin-B2 is intrinsic to the circulatory system or, rather, is exerted indirectly in other tissues. Further complicating the picture is the fact that this ligand can interact with multiple EphB class receptors (Gale et al., 1996), which are expressed in both vascular and nonvascular tissues (Adams et al., 1999). This suggested that a genetic identification of the relevant receptor(s) for ephrin-B2 in angiogenesis would be complicated by genetic redundancy. Consistent with this expectation, mutations in *EphB2*, which is expressed by nonvascular cells, and *EphB3*, which like EphB4 is xpressed on veins, individually had no phenotype, while *EphB2; EphB3* double mutants exhibited variable cardiovascular abnormalities with only 30% penetrance (Adams et al., 1999).

H r we have generated a targeted mutation in *EphB4*, introducing a tau-lacZ marker into the locus to

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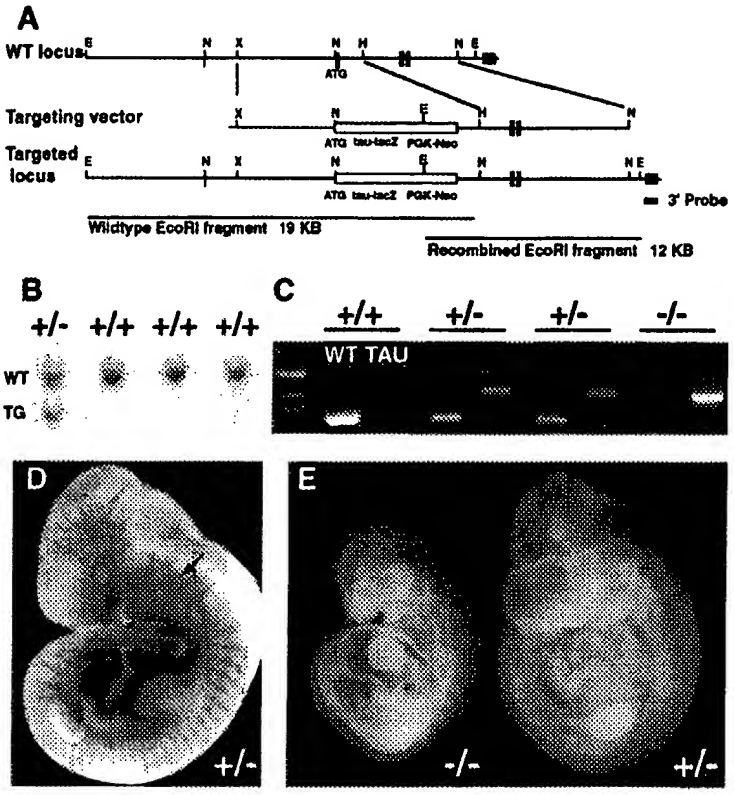

Figure 1. Targeting of the *EphB4* Locus

(A) Restriction maps of the wild-type (WT) *EphB4* locus, the targeting vector, and the targeted locus. The targeting vector contains a *tau-lacZ* reporter gene fused in-frame with the initiator codon of *EphB4*, which replaces the signal peptide–encoding exon, preventing membrane insertion of the receptor. A similar targeting strategy was previously used to inactivate *ephrin-B2* (Wang et al., 1998).

(B) Confirmation of germline transmission of the correct targeting event by Southern blotting of the progeny of an $EphB4^{tautacZ}$ heterozygote × C57Bl6/J cross. The genomic DNA has been restricted with EcoRI. WT, wild-type locus (19 kb; see [A]); TG, targeted locus (12 kb; see [B]).

(C) Genotyping of E9.5 progeny of an $EphB4^{tautacZ/+}$ intercross. WT, primers for the wild-type locus; TAU, primers for the targeted locus. Each sample was independently amplified with both primer sets.

(D) Whole-mount X-Gal staining of an E10.5 $EphB4^{tautacZ}$ heterozygous embryo. Expression appears restricted to the cardiovascular system. The arrow indicates the anterior cardinal vein (ACV).

(E) Morphologic appearance of an $EphB4^{tautacZ/tautacZ}$ embryo (−/−) at E10. Note the retarded overall growth and arrested cardiac development compared to the heterozygous littermate (+/−).

better visualize the expression of this gene. Surprisingly, unlike the broadly expressed *ephrin-B2*, *EphB4* is uniquely expressed in vascular endothelial and endocardial cells. This analysis also confirms that *EphB4* is preferentially expressed on veins. Remarkably and unexpectedly, the phenotype of homozygous *EphB4* mutants is virtually symmetric with that of *ephrin-B2* mutants. These data identify EphB4 as the major essential interaction partner of ephrin-B2 in angiogenesis and further indicate that the requisite function of this receptor is intrinsic to the circulatory system. Furthermore, the symmetry of the mutant phenotypes and the largely complementary expression of ephrin-B2 and EphB4 on blood vessels are consistent with the idea (Holland et al., 1996) that these molecules can participate in bidirectional signaling. Thus, these data provide further support for the concept that ephrin-B2 and EphB4 mediate reciprocal interactions between arteries and veins that are essential for proper angiogenic remodeling of the capillary beds.

Results

EphB4 Is Expressed Exclusively within the Cardiovascular System and Is Enriched on Veins Relative to Arteries

We (Wang et al., 1998) and others (Adams et al., 1999) previously provided evidence via in situ hybridization that *EphB4* mRNA is expressed on veins but n t arteries. However, because of the relatively low level of expression of this gen , and the high background obtained with the in situ hybridization probes, it was not clear wh ther *EphB4* was restricted to the cardiovascular system or whether, like *ephrin-B2* (Bennett et al., 1995; Bergemann et al., 1995; Wang and Anderson, 1997), it is expressed in many other nonvascular tissues as well. To provide a clearer picture of the pattern of *EphB4* expression, therefore, we inserted a *tau-lacZ* reporter gene (Mombaerts et al., 1996) into the *EphB4* targeting vector (Figure 1A; see Experimental Procedures).

Analysis of $EphB4^{tautacZ}$ expression in heterozygotes by whole-mount staining for β-galactosidase activity revealed apparently exclusive expression in the vasculature at E10 (Figure 1D). The vessels of the head (Figure 2A), intersomitic vessels (Figure 2C), and main trunk of the anterior cardinal vein (ACV; Figure 1D, arrow) were particularly apparent. Expression was also strong in the heart, especially in the ventricles (Figure 2E). At E8.75, expression was detected in the developing ACV (Figure 2G, open arrow), in the main trunk of the vitelline vein (Figure 2G, arrow), and in the yolk sac (Figure 2G, YS).

To determine more precisely the cellular localization of $EphB4^{tautacZ}$ expression within the cardiovascular system, we stained sections of heterozygous embryos using antibodies to β-galactosidase and markers of endothelial and smooth muscle cells. β-galactosidase expression colocalized with expression of PE-CAM, a pan-endothelial marker (Figures 2B, 2D, and 2I), and not with expression of alpha–smooth muscle actin (Figure 2H and data not shown), confirming that *EphB4* is expressed by endothelial and not by smooth muscle cells at these stages of devel pment. Similarly, within th heart, $EphB4^{tautacZ}$ was coexpressed with PE-CAM in endocardial cells (Figure 2F) and was not detect d in th myocardial layer (not sh wn).

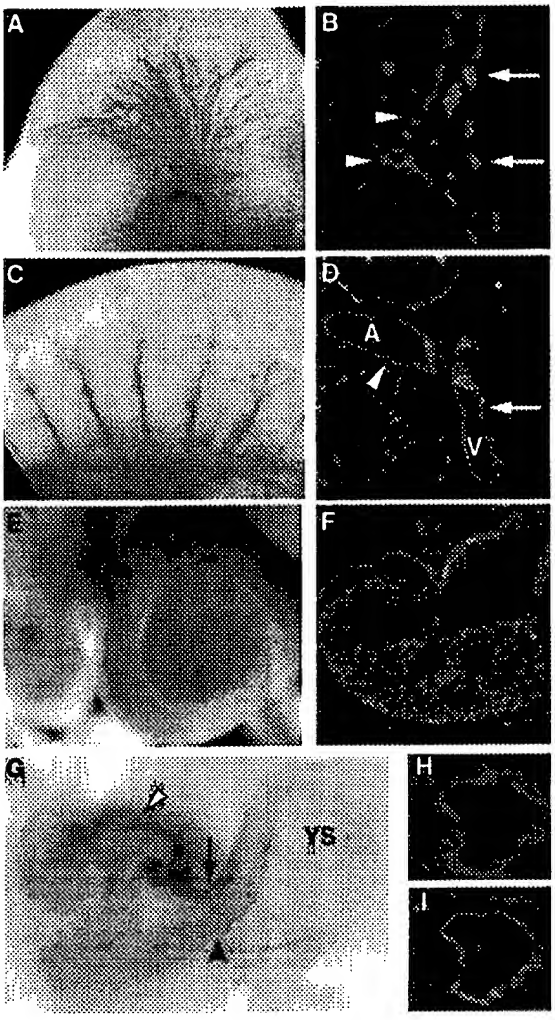

Figure 2. Embryonic Expression of *EphB4*$^{tau lacZ}$ Is Restricted to Endothelial Cells and Enriched in Veins

(A)–(F) are taken from E10 heterozygous embryos. (A and B) Head region. (A) Whole-mount X-Gal staining reveals expression in the branches of the ACV. (B) Double immunolabeling with antibodies to the pan-endothelial marker PE-CAM (green) and β-galactosidase (red) reveals coexpression of *EphB4*$^{tau lacZ}$ in the endothelial cells of the laterally located ACV branches (seen in cross section; arrows), but not in the medially located branches of the internal carotid artery (arrowheads). (C and D) Trunk region. (C) Expression in the intersomitic vessels. (D) Expression in the posterior cardinal vein (V, arrow) but not the dorsal aorta (A, arrowhead). (E and F) Heart. (E) Expression in the atrium and ventricle. (F) Coexpression with PE-CAM indicates expression in endocardial and not myocardial cells.

(G) E8.75 heterozygous embryo. Arrow, vitelline vein; arrowhead, vitelline artery; YS, yolk sac.

(H and I) E9.5 yolk sac vasculature triple-labled for β-galactosidase (red), alpha-smooth muscle actin (blue), and PE-CAM (green). (H) No overlap between *EphB4-tau-lacZ*-expressing and smooth muscle cells is observed. (I) Overlap with PE-CAM expressing cells is observed.

This analysis also confirmed that *EphB4* is preferentially expressed on veins. In the head, for example, expression overlapped with PE-CAM in the laterally located branches of the ACV (Figure 2B, arrows), but not in the medially located branches of the internal carotid artery (ICA; Figure 2B, arrowheads; s e also Figures 3E and 3F). Similarly, in the trunk region *EphB4* was xpressed in the posteri r cardinal vein (Figure 2D, arrow) but not in the dorsal aorta (Figure 2D, arrowhead). Similarly, in the yolk sac at E8.75 expression was detected in the vitelline vein (Figure 2G, arrow), but not the vitelline artery (Figure 2G, arrowhead). At E9.5, however, scattered, punctate expression of β-galactosidase was apparent in the branches of the vitelline artery (see Figures 7B and 7C), and overdevelopment of the X-Gal staining reaction revealed a low level of expression in other arteries as well that is below the detection limit of immunofluorescence (data not shown). In contrast, *ephrin-B2* appears to be absolutely arterial specific within the cardiovascular system (Wang et al., 1998). Nevertheless, *EphB4* is much more abundantly expressed in veins than in arteries and, unlike ephrin-B2, appears restricted to the cardiovascular system in embryos.

ephB4$^{tau lacZ}$ Homozygotes Display Defective Cardiovascular Development and Embryonic Lethality

Although our original study failed to detect venous expression of receptors other than EphB4 (Wang et al., 1998), a subsequent study detected expression of EphB3 in addition to EphB4 in veins (Adams et al., 1999). The reason for this discrepancy is not clear but may reflect a lower sensitivity of our in situ hybridization procedure. Whatever the explanation, these observations suggested that the function of EphB4 in veins might be redundant with that of EphB3 and therefore that little or no phenotype would be observed in *EphB4* knockouts. Indeed, no cardiovascular phenotypes were detected in *EphB3* single mutants (Adams et al., 1999).

To our surprise, embryos homozygous for the *EphB4*$^{tau lacZ}$ allele displayed cardiovascular defects and embryonic lethality with very high penetrance. By E9.5–E10, growth retardation, arrested heart development (see also Figure 6 below), and lack of blood flow were obvious in homozygous embryos (Figure 1E, −/−). Moreover, the recovery of homozygous *EphB4*$^{tau lacZ/tau lacZ}$ embryos at this age was well below expected Mendelian proportions (17%; n = 47 embryos examined). By E10.5, degeneration and necrosis were apparent throughout the embryo. The earliest overt morphologic defects were seen at E8.75–9.0, when heart looping appeared incomplete in some homozygous embryos (17%, n = 6 homozygotes from three litters examined). However, heartbeat and blood flow were still detectable in many homozygous embryos at E8.75–E9.0, and homozygotes were recovered at close to the predicted Mendelian ratio (23%; n = 26 embryos examined). No apparent defects were detected in heterozygous embryos (Figure 1E, +/−) compared to wild type (data not shown).

The *EphB4* Mutation Affects Morphogenesis of B th Arteries and Veins

The development of the peripheral circulatory system was examined in mutant and wild-type embryos by whole-mount staining with antibodies to PE-CAM. At E9.5-E10, th fine branches f th head vasculature

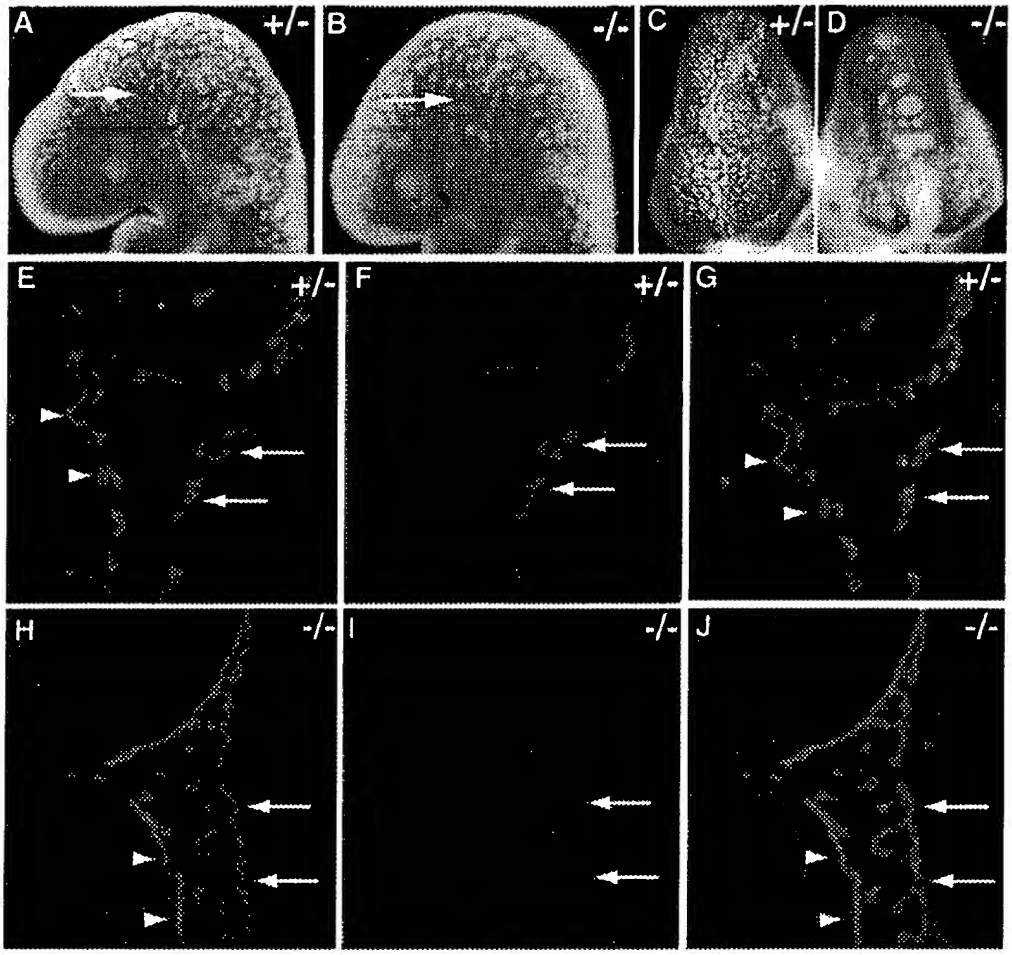

Figure 3. Defective Head Angiogenesis in *EphB4* Homozygotes

E9.5 embryos are shown. (A–D) Whole-mount PE-CAM staining reveals defective remodeling of the major head vessels, and fusion of the capillary network (B and D). Different specimens are shown in (B) and (D). (E–J) The *EphB4* mutation affects remodeling of both arteries and veins. Sections through $EphB4^{tautacZ/-}$ (E–G) and $EphB4^{tautacZ/tautacZ}$ (H–J) heads double labeled for PE-CAM (E and H) and β-galactosidase (F and I). A merged image of the two stains is shown in (G) and (J). Capillary fusion (reflecting arrested remodeling) of the branches of both the anterior cardinal vein (E versus H, arrows) and the internal carotid artery (E versus H, arrowheads) is evident in the mutant. Note that arteries and veins appear equally affected (E and H), even though the level of EphB4 expression is much higher in veins (F, arrows). The decreased expression of β-galactosidase in the mutant (F versus I, arrows) is characteristic and may reflect positive autoregulation by EphB4.

visible in heterozygous embryos (Figure 3A, arrow; Figure 3C) were not seen in homozygotes, where extensive fusion of the capillary network was visible (Figures 3B and 3D). This phenotype is suggestive of an arrest of the remodeling of the primitive, dilated vessels of the head plexus into smaller branched capillaries. Importantly, double labeling of sections with antibodies to PE-CAM and β-galactosidase to distinguish arteries (PE-CAM$^+$, β-gal$^-$) from veins (PE-CAM$^+$, β-gal$^+$) revealed fused vessels in both the branches of the anterior cardinal vein (cf. Figures 3E, 3G versus Figures 3H, 3J, arrows) where EphB4 expression is detectable in heterozygotes (Figure 3F, arrows), and also in the neighboring branches of the internal carotid artery (cf. Figures 3E, 3G versus 3H, 3J, arrowheads) where EphB4 expression in heterozygotes is low or undetectable by immunostaining (cf. Figure 3E, arrowheads, versus Figure 3F). The level of $EphB4^{tautacZ}$ expression in veins in homozygotes was typically much lower than in heterozygotes (Figures 3F and 3I, arrows). The reason for this is not yet clear but may reflect a positive autoregulatory function of EphB4. Whatever the case, these data indicate that *EphB4* function is similarly required for angiogenic remodeling of both the venous vessels of the head, where the gene is strongly expressed, and the arterial vessels where it is expressed at much lower levels.

An especially prominent feature of the *EphB4* mutant phenotype was disrupted development of the main trunk of the ACV (Figures 4A and 4B, arrows). Cross sections revealed that the luminal diameter of th ACV was reduced and that the vessel appeared split into multipl branches (Figures 4C and 4D, arrows). This ph notyp is virtually identical to that observed in *ephrin-B2* mutants (Adams et al., 1999; our own unpublish d data) and

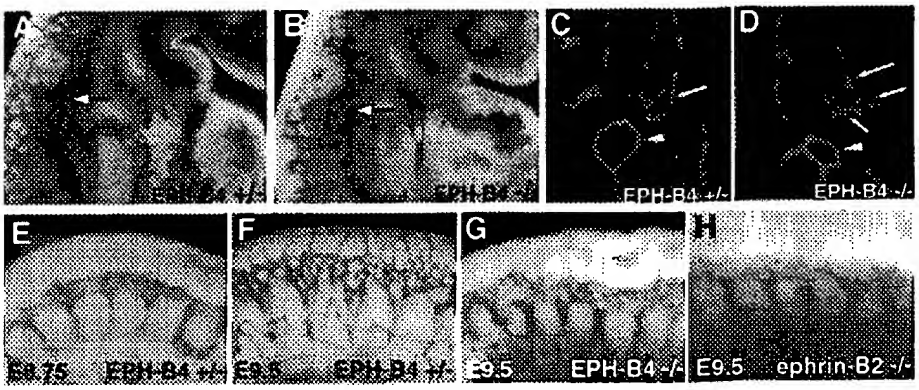

Figure 4. Defective Trunk Angiogenesis in *EphB4* Homozygotes

E9.5 embryos are shown.

(A and B) Whole-mount PE-CAM staining reveals defective formation of the main trunk of the anterior cardinal vein (arrow; cf. Figure 1D, arrow).

(C and D) Double-labeling for PE-CAM (green) and β-galactosidase (red) reveals that the ACV is split into multiple branches in the mutant (D, arrows), while the dorsal aorta appears unaffected (arrowheads).

(E–H) Whole-mount PE-CAM staining of intersomitic vessels. At E9.5, the remodeling of the vessels seen in heterozygotes (F) is defective in EphB4 homozygotes (G) and resembles an arrest at an earlier stage of normal development (E). (H) An *ephrin-B2* homozygous embryo at the same stage is shown for comparison. Note the similarity of the phenotypes in (G) and (H).

appears to reflect an arrest of remodeling of multiple small vessels into the single, large ACV vessel. In contrast, the dorsal aorta appeared relatively unaffected (Figures 4C and 4D, arrowheads), as was observed previously in the *ephrin-B2* mutant ([Wang et al., 1998]; however, an independently generated *ephrin-B2* knockout displayed variable defects in dorsal aorta formation [Adams et al., 1999]).

Defects in the development of both arterial and venous intersomitic vessels were also observed in both *EphB4* and *ephrin-B2* mutants. Whereas in heterozygous E9.5 embryos a finely anastomosed network of vessels was detectable with branches extending into and sometimes crossing the dorsal midline (Figure 4F), in both *EphB4* and *ephrin-B2* mutants this network appeared fused and truncated (Figures 4G and 4H). This appearance was very similar to that observed in heterozygous embryos at E8.75 (Figure 4E), suggesting a developmental arrest of angiogenesis in these vessels. Interestingly, *ephrin-B2* is expressed in caudal half-somites (Krull et al., 1997; Wang and Anderson, 1997), and it has been suggested that defective angiogenesis of intersomitic vessels in *ephrin-B2* mutants could reflect signaling from somitic cells to endothelial cells rather than between arteries and veins (Adams et al., 1999; Gale and Yancopoulos, 1999). However, reexamination of our *ephrin-B2*$^{tau lacZ}$ heterozygous embryos revealed clear expression of ephrin-B2 in arterial intersomitic vessels as well (data not shown). Interestingly, the ingrowth of these nascent vessels between the somites appeared to initiate just as expression of β-galactosidase in the caudal half-somites was fading. Since the arrest of remodeling by the *ephrin-B2* mutation occurs at an even later stage of intersomitic vessel maturation (E9.0–E9.5), these data indicate that *ephrin-B2* is no longer expressed in somites at th time that its function in angiogenesis is required.

Defects in Peripheral Angiogenesis in *EphB4* Mutants Are Observed in Mutant Embryos Exhibiting Heartbeat and Blood Flow

It was important to determine whether or not the peripheral angiogenic defects observed in *EphB4* mutant embryos were secondary to cardiac defects resulting in defective blood flow (see below). Because angiogenic remodeling in many areas is not obvious until after the onset of heartbeat (E8.0– E8.5; 10–13 somites [S]), it is often difficult to identify angiogenic defects at stages before blood flow is established. As an alternative, therefore, we exploited the variability in the time of onset of cardiac defects in early *EphB4* mutant embryos. Absent or defective heartbeat (detected by poor or absent erythrocyte movement in the heart and outflow tracts despite rhythmic myocardial contractions) was apparent in virtually all embryos by E9.5 (see Figure 6, below). We therefore asked whether any defects in peripheral angiogenic remodeling were visible prior to this stage in mutant embryos with manifestly normal cardiovascular function.

Embryos were collected between E8.75 and E9.0 (15–18S) and individually scored for the presence of heartbeat and blood flow (detected by the movement of erythrocytes through the embryonic vasculature), prior to genotyping. Following whole-mount PE-CAM staining, we asked whether any peripheral angiogenic defects were observable in those homozygous embryos that had exhibited apparently normal heartbeat and blood flow prior to fixation. Four homozygous embryos examined at 15S–25S showed normal blood flow and heartbeat but exhibited defects in angiogenesis of the head and ACV; three of these are illustrated in Figure 5. A 15S embryo exhibited defective formation of the head vasculatur (Figures 5A and 5B, arrows) and ACV (Figures 5A and 5B, open arrowheads), similar to those documented in later embryos (cf. Figures 3A and 3B, and 4A and 4B). No apparent defects in cardiac morph logy

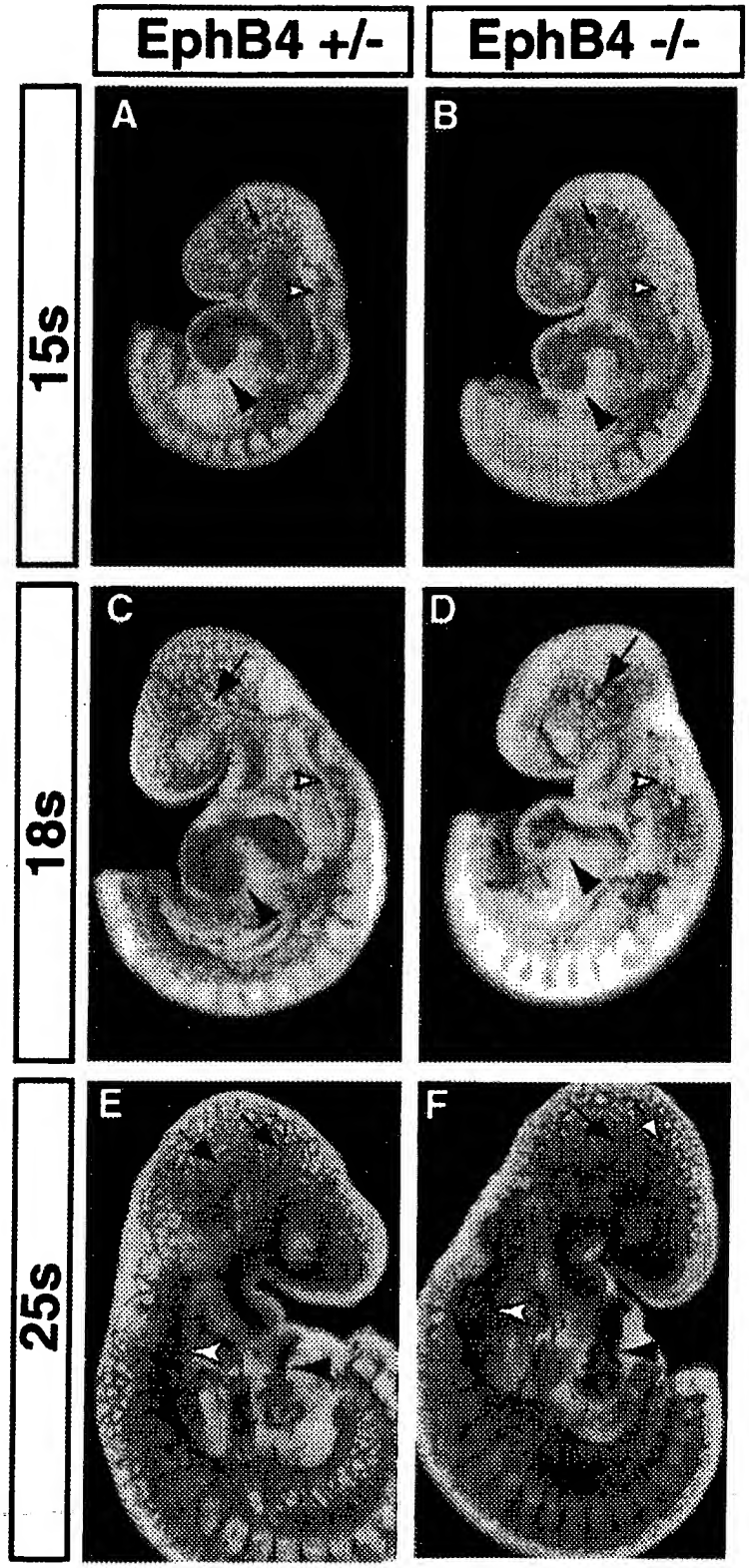

Figure 5. Angiogenic Defects Are Visible in Mutant Embryos Exhibiting Heartbeat and Blood Flow

Comparison of homozygous mutant (B, D, and F) and wild-type or heterozygous (A, C, and E) embryos by whole-mount PE-CAM staining at E8.75–E9.0. The actual stage of the embryos was determined by counting the number of somites (S) and is the same for normal and mutant littermates. All of the mutant embryos shown exhibited heartbeat and visible blood flow prior to fixation. Note the presence of defects in head vessel remodeling (arrows) and ACV formation (open arrowheads) in all of the mutant embryos (B, D, and F). Staining of the heart endocardium (closed arrowheads) appears normal in all embryos except (D); nevertheless, cardiac function was still apparent in this specimen.

were visible in this specimen (Figures 5A and 5B, cl sed arrowheads). Even more striking defects in angiogenesis of the head capillaries were observed in an 18S mutant embryo (Figures 5C and 5D, arrows), xcept that in this specimen a mild retardation of cardiac development was observed by PE-CAM staining (Figures 5C and 5D,

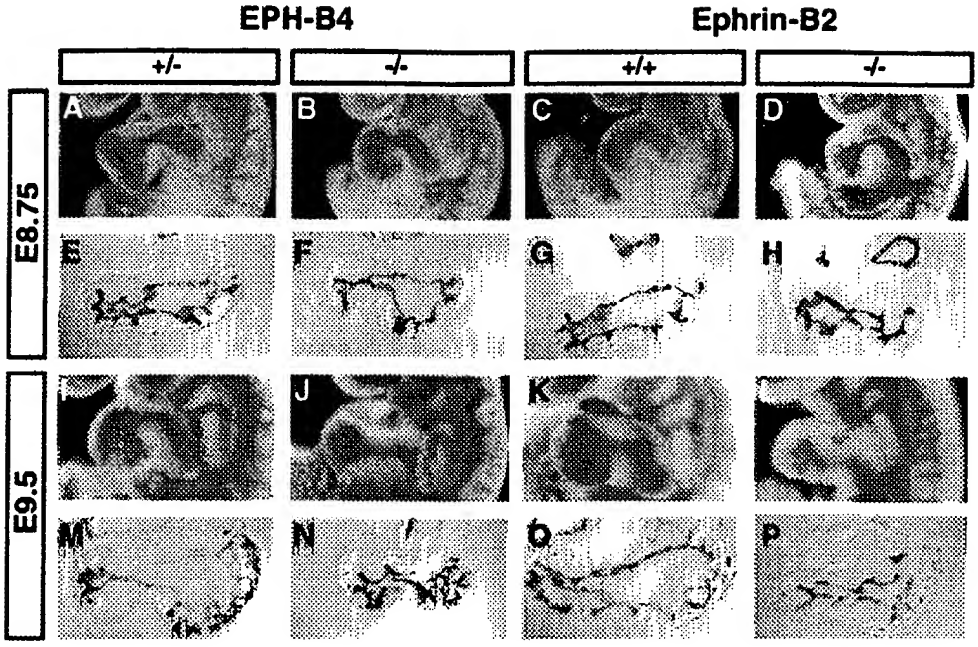

Figure 6. Arrested Cardiac Morphogenesis in Both *EphB4* and *ephrin-B2* Mutant Embryos

Anti-PE-CAM-stained embryonic hearts at E8.75 (A–H) or E9.5 (I–P) are shown in whole-mount (A–D and I–L) or cryosection (E–H and M–P) from both *EphB4* homozygous (B, F, J, and N) and *ephrin-B2* homozygous (D, H, L, and P) embryos. Normal littermate controls are shown for both mutants. Note that heart development appears normal at E8.75, but growth of the ventricular endocardium, like that of the heart itself, appears arrested at E9.5 (J, N, L, and P). At E10–E10.5, a more pronounced failure of myocardial trabeculation was apparent in the mutants (Wang et al., 1998; data not shown). Note that the phenotypes of the two mutants are virtually indistinguishable.

closed arrowheads); nevertheless, this retardation was evidently insufficient to prevent heartbeat and circulation at this stage. Finally, a 25S embryo showed defective ACV morphogenesis (Figures 5E and 5F, open arrowheads) despite a morphologically normal heart (Figures 5E and 5F, closed arrowheads). More subtle defects in head capillary angiogenesis were also apparent, upon close examination as well (Figures 5E and 5F, arrows).

These data indicate that although peripheral angiogenic defects in E9.5 *EphB4* homozygous mutant embryos are accompanied by defective cardiac development and consequent lack of blood flow, similar phenotypes can clearly be observed in earlier embryos in which such cardiac defects are not yet apparent. Taken together with the expression of *EphB4* and *ephrin-B2* in the peripheral vasculature, these observations support the idea that the angiogenic phenotype observed in the mutants reflects a requirement for a peripheral action of this ligand-receptor pair.

EphB4 Is Required for Proper Cardiac Development

To assess more carefully the cardiac phenotype of *EphB4* mutants and to compare it directly to that of *ephrin-B2* mutants, the hearts of homozygous embryos from both mutant lines were examined by whol -m unt PE-CAM staining and subsequent sectioning. As mentioned above, obvious d fects in cardiac morphogenesis or function were rare in homozygotes prior to E9.0 (Figures 6A–6D). This was confirmed by analysis of the ndocardium in sections (Figures 6E–6H). B tween E9.0 and E9.5, a retardation or arrest of cardiac morphogenesis appeared to occur in *EphB4* homozygotes. The heart failed to increase in size, cardiac looping was incomplete, and the endocardium failed to expand (Figures 6I, 6J, 6M, and 6N). Very similar defects were observed in *ephrin-B2* homozygotes at these stages (Figures 6K, 6L, 6O, and 6P). By E10, a clear failure of myocardial trabeculation was apparent in those few homozyotes that survived (Wang et al., 1998; data not shown). Thus, the phenotype of the *EphB4* and *ephrin-B2* mutants in cardiac development is almost indistinguishable and appears to reflect a requirement for these genes in growth and morphogenetic events that occur 12–24 hr after the initiation of heartbeat.

EphB4 Is Required for Angiogenic Remodeling in the Yolk Sac

ephrin-B2 is required for angiogenic remodeling of the yolk sac on both the arterial (posterior) and venous (anterior) sides (Wang et al., 1998; Adams et al., 1999). Similarly, phenotypic defects were revealed by PE-CAM staining of E9.5 *EphB4* homozygous mutant embryos in both the arterial (Figures 7F and 7G) and venous (Figures 7H and 7I) domains. As in the case of the *ephrin-B2* mutant, the phenotype appeared to reflect an arrest at the primitive plexus stage, although this arrest appeared more severe on the ven us than n th arterial sides (Figure 7G and 7I). Because ext nsive angiogenic rem deling of the yolk sac has not yet occurred in E8.75–E9.0 embryos, it was difficult to detect obvious diff rences between mutant and wild-type tissue at these

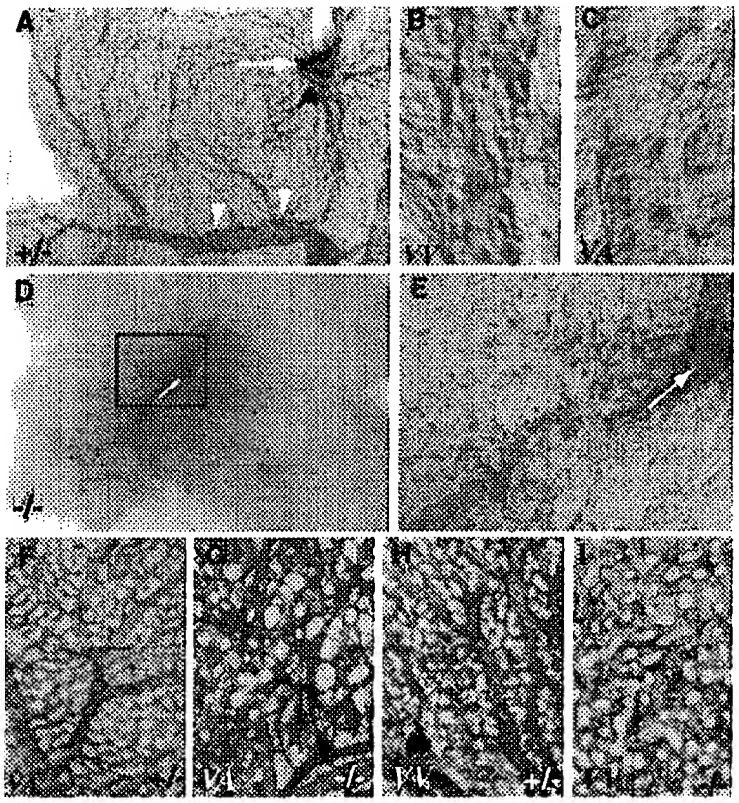

Figure 7. Defective Yolk Sac Angiogenesis in *EphB4* Mutants

(A–C) Whole-mount X-Gal staining of heterozygous embryos. Note that expression of the *EphB4*$^{tau lacZ}$ allele is enriched on the venous (A, arrow; B) relative to the arterial (A, arrowhead; C) side of the yolk sac. Note also the patchy nature of the expression; β-galactosidase-expressing cells in the vitelline artery (C) are surrounded by *EphB4*-nonexpressing cells. By contrast, expression of *ephrin-B2* in the yolk sac arteries is more homogenous (Wang et al., 1998).

(D and E) Expression of β-galactosidase in homozygous mutants at E9.5. Note the scattered, punctate nature of the staining, and reduced intensity relative to the heterozygote (A). (E) represents a higher magnification view of the boxed area in (D). Arrow indicates the entry point of the vitelline vein. Note the increased concentration of β-gal$^+$ cells in this region.

(F–I) Whole-mount PE-CAM staining of E9.5 yolk sacs reveals defective angiogenic remodeling on both the venous (H and I) and arterial (F and G) sides of *EphB4* mutant embryos. Defects appear slightly less severe on the arterial side. *VA*, vitelline artery; *VV*, vitelline vein.

ages. Therefore, we cannot completely exclude that some or all of the *EphB4* yolk sac phenotype is secondary to defective circulation. However, we did detect angiogenic defects in a yolk sac from at least one E8.75–E9.0 *ephrin-B2* homozygous mutant in which heartbeat and blood flow were detectable (data not shown).

To examine the effect of the *EphB4* mutation on the distribution of *EphB4*$^{tau lacZ}$-expressing cells on the yolk sac, we examined heterozygous and homozygous embryos by whole-mount X-Gal staining. Two surprising findings emerged from this analysis. First, unlike the case of *ephrin-B2*, whose expression is absolutely restricted to the arterial side of the yolk sac (Wang et al., 1998; Adams et al., 1999), expression of *EphB4*$^{tau lacZ}$ in heterozygotes while clearly strongest on the venous side (Figure 7A, arrow) was detected on the arterial side at a lower level as well (Figure 7A, arrowheads). Examination at higher magnification revealed, however, that this arterial expression of *EphB4*$^{tau lacZ}$ was not uniform, but rather patchy as if confined to individual cells or groups of cells (Figure 7C). Expression also appeared punctate on the venous side but was clearly more extensive than on the arterial side (Figure 7B).

The second unexpected finding concerned the distribution of *EphB4*$^{tau lacZ}$-expressing cells in homozygous mutant yolk sacs. In *ephrin-B2*$^{tau lacZ}$ homozygotes, expression of β-galactosidase is similar in intensity to that in heterozygotes and is distributed fairly uniformly throughout the primitive plexus on the arterial side (Wang et al., 1998). In contrast, expression of β-galactosidase in *EphB4* homozygotes is much weaker than in heterozygotes and is highly punctate and nonuniform, although it still appears most extensive on the venous side (Figure 7D). Interestingly, the distribution of labeled cells appeared graded, with the highest density toward the entry point of the vitelline vein (Figures 7D and 7E, arrows). Whether this reflects a migration of *EphB4*-expressing cells toward or away from the vitelline vein, or some other phenomenon, is under investigation.

Discussion

Ephrins and their receptors have recently emerged as essential regulators of angiogenesis in vivo, equal in genetic importance to other ligand-receptor systems such as the angiopoietins and VEGFs (reviewed in Gale and Yancopoulos, 1999). In contrast to these diffusible ligands that can act over many cell diameters, however, the ephrin system has evolved to mediate interactions between cells that touch each other. Furthermore, the restricted and often complementary expression of ephrins and their receptors (not only within the circulatory system but in other tissues as well [Gale et al., 1996]) suggests that these contact-dependent interactions often occur between dissimilar cell types. To understand the role of ephrins in angiogenesis, therefore, it is important to define the essential receptors for these ligands, the cell–cell interactions they control, and the cellular behaviors such interactions regulate.

EphB4 Is the Major Essential Rec pt r f r Ephrin-B2-Mediated Signaling during Cardi vascular D v l pment

The finding that ephrin-B2 is required for angiogenesis (Wang et al., 1998) left open the question of the functionally relevant receptor for this ligand. This question is

important, because ephrin-B2 can interact with multiple EphB class receptors (Gale et al., 1996), several of which are expressed on nonvascular tissues as well as on endothelial cells (Adams et al., 1999; Gale and Yancopoulos, 1999). The present data identify EphB4 as the major genetically essential receptor for ephrin-B2 in cardiovascular development. This in itself is surprising given the functional redundancy that has been demonstrated for other EphB receptors in angiogenesis (Adams et al., 1999). Based on such data, we would have predicted that the *EphB4* mutation on its own would yield little or no phenotype. The fact that it phenocopies the *ephrin-B2* mutation is quite unexpected and suggests that EphB4 is the predominant functional partner for ephrin-B2 in this system.

Why is the loss of EphB4 function not compensated by that of EphB3, which also can interact with ephrin-B2 (Gale et al., 1996) and is like EphB4 specifically expressed on veins (Adams et al., 1999)? First, the level of *EphB3* expression may simply not be high enough to compensate for the loss of EphB4 function; indeed, we have been unable to detect expression of this gene in veins by in situ hybridization (Wang et al., 1998). Second, EphB4 uniquely binds to ephrin-B2 among all ephrin-B family ligands (Brambilla et al., 1995; Sakano et al., 1996), while EphB3 is less specific (Gale et al., 1996). Activation of EphB4 by ephrin-B2 may therefore send a unique signal that is not mimicked by activation of EphB3. This could also explain why ephrin-B1, which is expressed on arteries (Adams et al., 1999) and which can interact with EphB3 (but not EphB4), evidently cannot compensate for the loss of ephrin-B2 (Wang et al., 1998). Nevertheless, the fact that *EphB2; EphB3* double mutants do have a partial phenotype indicates that these receptors must play some redundant role in vascular development, perhaps in mediating ancillary interactions between arteries and neighboring cell types, rather than between arteries and veins (Adams et al., 1999; Gale and Yancopoulos, 1999).

Ephrin-Mediated Signaling Is Intrinsic to the Developing Circulatory System

The fact that *EphB4* is essential for angiogenesis, taken together with the specificity of its expression, allows us to resolve the question of whether ephrin-B2-mediated signaling is actually required within blood vessels (Wang et al., 1998; Adams et al., 1999). Our tau-lacZ insertion reveals clearly that *EphB4* is exclusively expressed in the embryo by vascular endothelial cells. There is thus no ambiguity about whether the essential function of this receptor is exerted within the cardiovascular system. Given that EphB4 has no other specific ligand than ephrin-B2, that ephrin-B2 is specifically expressed on arteries and that the two mutations yield symmetrical phenotypes, these data also suggest that at least some functions of ephrin-B2 in angiogenesis are intrinsic to the circulatory system as well. Our results therefore not only reinforce the idea that ephrin-B2 and EphB4 are key regulators of angiogenesis, but also d monstrate that the requisite activity of EphB4 is intrinsic to the circulatory system.

An important question is whether the angiogenic remodeling defects seen in the *ephrin-B2* and *EphB4* mutants reflect a local function for these molecules in the peripheral vasculature, or rather are secondary to impaired blood flow caused by defectiv h art d v lopment. This confound is not unique to the ephrins but complicates the analysis of many mutants · xhibiting both cardiac and angiogenic phenotypes. In *EphB4* homozygotes, however, cardiac development appears normal up to about E9.0 and then seems to arrest (Figure 6). Nevertheless, we can clearly observe defective angiogenic remodeling in E8.75–E9.0 mutant embryos where heartbeat and blood flow are still detectable, that is, before the onset of the cardiac defects (Figure 5). While we cannot exclude that more subtle hemodynamic alterations cause the phenotype, the fact that both ligand and receptor are actually expressed in the peripheral vasculature further argues that their functions are likely required there. Selective rescue of the mutants in endocardial cells, or selective knockout in endothelial cells, could in principle resolve this issue but is currently precluded by the lack of appropriately specific promoters.

The Cellular Function of Ephrin Signaling in Angiogenesis

The cellular function of ephrin-mediated signaling in angiogenesis remains unclear. Although ephrins have been shown to function as repulsive guidance molecules for growing axons (Drescher et al., 1995; Nakamoto et al., 1996) and migrating neural crest cells (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997), the evidence thus far in the circulatory system suggests that they promote endothelial cell migration (Pandey et al., 1995), capillary formation (Stein et al., 1998), and sprouting (Adams et al., 1999). However, in the circulatory system as in many other places in the embryo, ephrins and their receptors are reciprocally expressed at boundaries between dissimilar cell types or tissues (Gale et al., 1996). Recent functional studies have provided evidence that signaling by ephrin-B ligands and EphB receptors is important in the maintenance of such boundaries (Mellitzer et al., 1999; Xu et al., 1999). By analogy, interactions between ephrin-B2 and EphB4-expressing endothelial cells could play a role in the establishment or maintenance of the arteriovenous (A-V) boundary. If so, then the fact that the mutant phenotypes in both the *ephrin-B2*$^{-/-}$ and *EphB4*$^{-/-}$ capillary plexii extend beyond the A-V boundary into the capillary network would imply that proper boundary formation is essential for remodeling of the entire network to occur.

The Expression and Symmetrical Mutant Phenotypes of *EphB4* and *ephrin-B2* Provide In Vivo Genetic Evidence Consistent with Bidirectional Signaling

A great deal of interest has focused on the possibility that ephrin-B class transmembrane ligands may also function as signal-transducing receptors, mediating bidirectional signaling at boundaries between ephrin-B- and EphB-expressing cells. Consistent with this possibility, engagement of ephrin-B class ligands with their receptors results in tyrosine phosphorylation of the former on their cytoplasmic tails (Holland et al., 1996; Bruckner et al., 1997). Although the biological consequences of this phosphorylati n were not established, recent gain-of-function experiments in zebrafish have shown that the cytoplasmic domain of ephrin-B class ligands is required to promote segregation fr m EphB

receptor-expressing cells in vivo (Mellitzer et al., 1999; Xu et al., 1999).

A prediction of the bidirectional signaling model is that loss-of-function mutations in an ephrin-B class ligand and its cognate EphB class receptor, expressed on complementary and interacting cell populations, should produce symmetrical phenotypes of both an autonomous and a nonautonomous nature. The present data provide a striking example of such symmetrical loss-of-function phenotypes. That mutations in a ligand or its receptor yield similar phenotypes does not in and of itself prove that bidirectional signaling occurs. Nevertheless, the fact that the *EphB4* mutation causes a similar phenotype in arteries as does the *ephrin-B2* mutation, taken together with the preponderant expression of the receptor on veins, is consistent with the idea that reciprocal signaling occurs. In support of this idea, targeted deletion of the EphB2 receptor caused axon guidance defects in an apparently non–cell autonomous manner (Henkemeyer et al., 1996). This function was independent of the EphB2 tyrosine kinase domain, suggesting that EphB2 activates an ephrin-B class ligand expressed on axons. However, no mutation in any such ligand has yet been identified that phenocopies this *EphB2* mutant.

There are two caveats associated with this interpretation, however. First, the low-level, patchy expression of EphB4 in arteries leaves open the possibility that this receptor functions autonomously in arteries, in which case a reverse signaling function for ephrin-B2 could not necessarily be inferred. However, an autonomous requirement for EphB4 function in arteries seems inconsistent with the similar strengths of the arterial and venous *EphB4*$^{-/-}$ phenotypes (cf. Figure 3G versus 3J), given the striking difference in EphB4 expression levels between the two vessel subtypes (Figures 3E and 3F). Second, even if the arterial requirement for EphB4 is exerted nonautonomously in veins, it is still formally possible that it is mediated indirectly, for example, by mechanical forces or by an unknown ligand whose expression or secretion are EphB4 dependent, rather than by reverse activation of ephrin-B2. However, the fact that EphB4 binds tightly and specifically to ephrin-B2, taken together with the receptor-induced tyrosine phosphorylation of ephrin-B cytoplasmic domains (Holland et al., 1996; Bruckner et al., 1997), argues that bidirectional signaling is the most likely explanation for the symmetrical mutant phenotypes.

Ephrin-B2 and EphB4 as Potential Targets of Angiogenic Therapy

Our results establish that the interaction between ephrin-B2 and EphB4 is indispensable for embryonic angiogenesis. This raises the question of whether it is required in settings of adult neovascularization as well. Preliminary observations indicate that expression of this ligand-receptor pair persists in the adult cardiovascular system and that ephrin-B2 at least is expressed during tumor angiogenesis (D. Shin, and G. Garcia-Card nas et al., unpublished observations). Pharmacologic p rturbation of other signaling systems genetically required for embryonic angiogenesis, such as angiopoietin1-tie2, effectively inhibits tumor vessel formation (Folkman, 1998a; Goldman et al., 1998; Lin et al., 1998). By analogy, functional perturbation of ephrin-B2–EphB4 signaling may provide a similar but alternative strategy for antiangiogenic cancer chemotherapy as well. Furthermore, the vessel-specific xpression of these molecules may permit novel approaches to proangiogenic therapies (Folkman, 1998b; Schumacher et al., 1998) directed selectively at arteries or veins.

Experimental Procedures

Targeted Disruption of the *EphB4* Gene

A 156 bp probe starting from the ATG of the mouse *EphB4* gene was used to screen a 129SVJ genomic library (Stratagene). Analysis of overlapping clones revealed that the first exon, including the signal sequence, ended 50 bp after the ATG. To construct a targeting vector, a 2.75 kb Xba1-NcoI fragment whose 3′ end terminated at the ATG was used as the 5′ arm. A 5.3 kb *tau-lacZ* coding sequence (Mombaerts et al., 1996) was fused in-frame after the ATG. An NcoI-NcoI fragment encoding 5 Myc epitope tags was PCR amplified from pCS2+MT (Turner and Weintraub, 1994) and inserted at the ATG, resulting in a 5 Myc-Tau-lacZ fusion. A LoxP site–flanked PGKneo gene (the generous gift of J. Yoon and B. Wold) derived from pPNT (Tybulewicz et al., 1991) was inserted downstream of the *tau-LacZ* gene. For the 3′ arm, a PCR fragment extending 500 bp downstream of the end of the first coding exon to the nearest HindIII site was inserted immediately downstream of the PGKneo cassette, and then ligated at its 3′ end to a 5.5 kb HindIII-NcoI fragment. This resulted in deletion of only the first coding exon, leaving all downstream intronic regions intact. Normal (19 kb) and targeted (12 kb) loci are distinguished by EcoRI digestion when probed with a 380 bp PCR fragment representing the fourth coding exon (Figure 1A). Electroporation, selection, and blastocyst injection of AB-1 ES cells (strain 129 SvJ) were performed essentially as described (Ma et al., 1998), with the exception that FIAU selection was omitted. ES cell targeting efficiency via G418 selection was 1 out of 8 clones. Germline transmission of the targeted *EphB4* locus was confirmed by Southern blotting (Figure 1B). Primers for the Southern probe are 5′-GCAGAACATCTGACTCGGAAGC-3′ (5′) and 5′-CTCTGCATACTTTGTTGCTTTCC-3′ (3′). Subsequent genotyping was done by genomic PCR. A 5′ primer in the 5′ UTR of *EphB4*, 5′-ATCGTTGAGAGGCCCTCGAC-3′ (5′), was used for both wild-type (235 bp product) and targeted (350 bp product) loci. The 3′ primers for detecting wild-type and targeted loci are 5′-GTGCTATT GGTCCGAAGTGTT-3′ (3′), downstream of the first coding exon, and 5′-CTGAGCATGATCTTCCATCAC-3′ (3′) in the *tau-lacZ* gene, respectively. Germline chimeras were backcrossed onto a pure C57Bl6/J background, and all subsequent breeding was on this background, exactly as performed for the *ephrin-B2* mutation we described previously (Wang et al., 1998).

LacZ and Immunohistochemical Staining

For LacZ staining, embryos and yolk sacs were removed between E8.75 and E10.0, fixed in 0.25% glutaraldehyde/PBS for 5 min, rinsed twice with PBS, and stained for 1 hr to overnight at 37°C in X-Gal buffer (1.3 mg/ml potassium ferrocyanide, 1 mg/ml potassium ferricyanide, 0.2% Triton X-100, 1 mM MgCl2, and 1 mg/ml X-Gal in PBS [pH 7.2]). LacZ-stained embryos were postfixed prior to being photographed. For antibody staining, embryos were first fixed overnight in 4% paraformaldehyde/PBS at 4°C. Embryos were embedded in 15% sucrose and 7.5% gelatin in PBS, and sectioned on a cryostat at 15 μm. Procedures for whole-mount staining with anti-PECAM-1 antibody (clone MEC 13.3, Pharmingen, 1:300 overnight at 4°C) were done essentially as described (Wang et al., 1998). HRP-conjugated secondary antibodies (Jackson, 1:300, overnight at 4°C) were used for all whole-mount PECAM-1 stainings. For immunofluorescent detection of PECAM-1 on sections, secondary antibodies conjugated to FITC or Cy-5 (Jackson, 1:200) were applied for 1 hr at room temperature. For immunofluorescent detection of β-galactosidase, sections were stained with preabsorbed anti-β-galactosidase antibody (3-prime 5-prime, 1:1000) overnight at 4°C, followed by secondary antibody conjugated to Cy-5 (Jackson) for 1 hr at room temperature. Confocal microscopy was carried out on an LSM

510 (Zeiss). Anti-Smooth Muscle Actin antibody conjugated to Cy3 (Sigma, 1:250 overnight at 4°C) was used to detect α-Smooth Muscle Actin. For triple labeling experiments, PECAM, β-galactosidase, and α-Smooth Muscle Actin were detected using FITC, Cy5, and Cy3, respectively.

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